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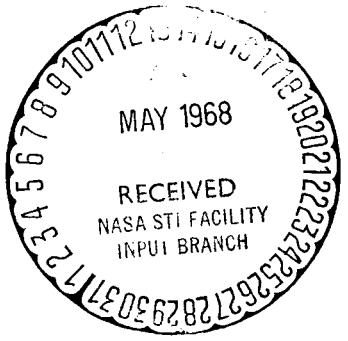
Re-order No. 67-115



EXPERIMENTAL ASSEMBLY & STERILIZATION LAB

IDENTIFICATION of MICROBIOLOGICAL ISOLATES

15 April 1967
Task 5.4
JPL CONTRACT 951624

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Prepared by

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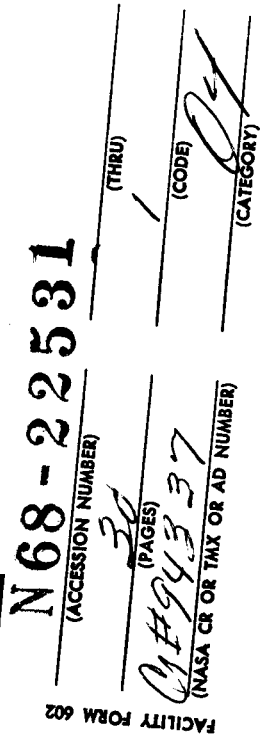
for

JET PROPULSION LABORATORY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA



-NOTE-

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EXPERIMENTAL ASSEMBLY & STERILIZATION LAB

IDENTIFICATION
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TABLES

ABBREVIATIONS

B. B. L.	Baltimore Biological Laboratory
°C	Degrees Centigrade
mm	Millimeters
MR-VP	Methylred Voges-Prosaurer Reactor
S. A. B.	Society of American Bacteriologists
TSA	Trypticase Soy Agar

I. INTRODUCTION

Eighty-three isolates from the EASL facility and related environments were identified as to genus and species.

The approach for identification was three-fold, based on the procedures of Cowan and Steel (2) (Manual for the Identification of Medical Bacteria), Breed et al (1) (Bergey's Manual of Determinative Bacteriology), and possibly a procedure evolved by M. Favero (3), U.S.P.H.S., Phoenix, Arizona. The nomenclature used to describe the organisms was that of Bergey's Manual, 7th edition.

II. MATERIALS AND METHODS

A. EXPERIMENTAL PLAN

Scheme for the identification of JPL supplied EASL isolates was an idealized one. Additions and deletions of biochemical tests were required due to the experimental results obtained during the course of the study. Outlined below is the plan as executed:

1. Describe colonial characteristics and pigmentation.
2. Subculture isolate on TSA, incubate at 32°C for 18–24 hours aerobically.
3. Gram stain and record morphology such as
   a. Gram positive rods
   b. Gram negative rods
   c. Gram positive cocci
   d. Gram negative cocci
   e. Miscellaneous gram positive or negative vibrios or spirillae.
4. Gram positive rods.
   a. Subculture on sporulation medium
   b. Incubate and make smears of resultant growth
   c. Spore stain

d. Cells with spores are members of the Genus Bacillus or Clostridium

e. Describe morphology of the cell and spore; list position of spore in cell

f. Reserve specimens without spores for later study. (See Section I.)

g. Test for motility

h. Test for the following biochemical characteristics:

1) citrate utilization

2) gelatin hydrolysis

3) casein hydrolysis

4) starch hydrolysis

5) fermentation of glucose, arabinose and mannitol in a medium with an ammonium salt base

6) indole production

7) Voges-Proskauer reaction

8) nitrate reduction

9) urease

10) lecithovietellin reaction

i. Sporeforming specimens with poor or limited growth should be subcultured and incubated anaerobically

j. Improved growth indicative of members of the genus Clostridium that are not strict anaerobes

k. Suspected Clostridia should be assayed for meat digestion characteristics, effects on litmus milk, H_2S production, and fermentation characteristics of glucose, lactose, and sucrose

l. Non-spore-forming rods will be stained with Loeffler's alkaline methylene blue and examined for morphology

m. Rods that may be club shaped or swollen, arranged in palisade or picket fence formations, or show irregular staining (metachromatic granules or segmental staining) will be considered diptheorids.

n. Specimens that are not sporeformers and do not demonstrate the characteristics listed in subparagraph *l* after staining with Loeffler's alkaline methylene blue could be members of the following genera and will not be further examined: Kurthia, Erysipelothrix, Lactobacillus, Actinomyces, Norcardia, Mycobacterium, or other growths.

1) These organisms will be listed as gram positive, non-sporeforming rods (specimen 1, 2, 3, 4, etc.), and their colonial morphology given

5. Gram negative rods

a. Note colonial morphology and pigment production

b. Test for catalase, oxidase, glucose fermentation, oxidative-fermentation, motility, subculture onto potato plugs to enhance pigment production, and inoculate onto TSI (triple sugar iron Agar). Based on the results of these tests the specimens could be divided into the families Enterobacteriaceae, Pseudomonadaceae and Achromobacteraceae

c. Enterobacteriaceae

1) This family is divided into nine genera: Escherichia, Aerobacter, Klebsiella, Paracolobactrum, Erwinia, Serratia, Proteus, Salmonella and Shigella

2) If the organisms do not fit into the genera Escherichia, Aerobacter, Klebsiella, Serratia, or Proteus, they will be listed as gram-negative rods, non-spore forming, along with their biochemical characteristics, colonial morphology, and pigmentation, and given a code number

3) The following biochemical tests will be performed:

a) citrate utilization

b) growth in the presence of KCN

c) gelatin hydrolysis

d) fermentation of glucose, arabinose, lactose, sucrose, adonitol, dulcitol, mannitol, inositol

e) indole production

f) Voges-Proskauer test

g) H_2S production

h) urease activity

i) lysine, arginine and ornithine decarboxylase

j) phenylalanine conversion to phenylpyruvic acid

d. Pseudomanadaceae

1) The family is divided into twelve genera: Pseudomonas, Xanthomonas, Acetobacter, Aeromonas, Photobacterium, Azotomonas, Zymomonas, Protaminobacter, Aliginomonas, Mycoplana, Zoogloea, and Halobacterium

2) If organisms do not fit into the genus Pseudomonas, they will be listed as gram-negative non-spore-forming rods, along with their colonial morphology, pigment production, and biochemical characteristics, and given a code number

3) The following biochemical tests will be performed:

a) citrate utilization

b) growth in presence of KCN

c) gelatin hydrolysis

d) starch hydrolysis

e) mannitol fermentation

f) indole production

g) nitrate reduction

h) urease activity

i) arginine dihydrolase activity

e. Achromobacteraceae

1) The family is divided into five genera: Alcaligenes, Achromabacter, Flavobacterium, Agarbacterium, and Beneckea

2) If the organisms do not fit into the genera, Alcaligenes, Achromobacter, or Flavobacterium, they will be listed as gram-negative, non-spore-forming rods, along with their colonia morphology, pigment production, and biochemical characteristics.

3) The following biochemical tests will be performed:

a) action on litmus milk

b) gelatin liquification

c) indole production

d) nitrate reduction

e) urease activity

f) H_2S production

g) starch hydrolysis

h) fermentation of glucose, sucrose, lactose, maltose, mannitol, xylose

i) methyl red test

j) citrate utilization

6. Gram positive cocci

Gram positive cocci can be divided into the genera Micrococcus, Staphylococcus, Streptococcus, Gaffkya, and Sarcina, by the following morphological and biochemical tests:

a. Cells are found in irregular masses, occasionally in singles or pairs. If glucose is acted upon, it is by aerobic oxidation. Growth is aerobic and pigments may be produced, genus Micrococcus.

b. Cells are found in irregular masses, glucose fermented anaerobically with the production of acid, facultatively anaerobic, genus Staphylococcus

c. Cells normally occur in tetrad or packets, white to pale yellow chromogenesis, non-motile, nitrate not reduced and lactose fermented to produce acid, genus Gaffkya

d. Cells occur in packets, white, yellow, orange or red chromogensis and usually non-motile, genus Sarcina

e. Cells occur in pairs or chains, gelatin rarely liquified, catalase negative, colonies small, usually less than 1 mm in diameter, genus Streptococcus

f. For further identification of the members of the genus Micrococcus, the following biochemical tests will be run:

1) nitrate reduction

2) phosphatase activity

3) coagulase activity

4) Voges-Proskauer reaction

5) oxidation-fermentation reaction for glucose, sucrose, and lactose

6) litmus milk reactions

7) gelatin hydrolysis

8) catalase activity

g. For further identification of the members of the genus Gaffkya, the following biochemical tests will be run:

1) coagulase activity

2) mannitol fermentation

3) pigment production

h. For further identification of the members of the genus Sarcina, the following biochemical tests will be run:

1) fermentation reactions with glucose, fructose, sucrose, lactose, maltose, starch and cellulose

2) litmus milk reaction

3) urea utilization

4) pigmentation

7. Gram Negative Cocci

a. The Gram negative cocci of interest would be members of the genus Neisseria

b. Other Gram negative cocci, which do not fit into this genus, will be listed as gram-negative cocci, along with their biochemical characteristics, colonial morphology, and pigmentation, and given a code number.

c. The following biochemical characterics will be examined:

1) fermentation of glucose, lactose, maltose, fructose, sucrose, and mannitol

2) pigmentation production

3) nitrate reduction

4) catalase activity

8. Favero Approach

A possible alternate scheme of identification of isolates is outlined in the Appendix A.

B. CULTURE MEDIA AND REAGENTS

The experimental plan outlines possible strains, biochemical tests, and culture media that might be utilized for the identification of the EASL facility and related environments isolates.

The following strains, biochemical tests, and culture media were actually used:

1. Stains

a. Gram stain (Hucker's Modification -- 6) and spore stain (Wirtz's Method as modified by Bartholomew and Mittwer -- 6)

2. Media

   a. Trypticase soy agar (B. B. L.)

   b. 7-percent and 10-percent NaCl broth. NaCl in proper concentrations was added to nutrient broth (Difco)

   c. Dextrose agar, 1-percent dextrose, was added to trypticase soy agar (B. B. L.)

   d. Tyrosine agar, 0. 1-percent tyrosine, re-agent grade, was added to trypticase soy agar (B. B. L.)

   e. Soybean agar, 1-percent soytone (Difco), was added to trypticase soy agar (B. B. L.)

3. Biochemical tests

   a. Nitrate reduction: nitrate agar (Difco) was used and nitrate reduction tested according to S. A. B. Methods (6).

   b. Starch hydrolysis: starch agar, 1-percent soluble starch, was added to nutrient agar; hydrolysis determined by flooding with Lugol's iodine after 24 hours incubation at 32°C.

   c. Citrate utilization: Simmon's citrate medium (Difco) was used.

   d. Voges-Proskauer reaction: two media employed MR-VP medium Difco and Smith's (5) modified Voges-Proskauer medium

   e. Litmus milk reaction: litmus milk (Difco) was used

   f. Sugar fermentation: two types of media were used. For the gram-positive cocci, Difco's phenol red broth with appropriate carbohydrate disks were used. When examining the fermentation characteristics of the Bacillus genus, the Smith (5) modification of the Ayers, Rupp & Johnson medium was used as a base, plus the appropriate carbohydrates in disk form (Difco).

   g. Catalase reaction: H_2O_2 was used to detect catalase activity according to the S. A. B. Methods (6).

   h. Coagulase test: coagulase agar base (Difco) to which was added 7-percent sterile blood plasma.

III. RESULTS

Eighty-three specimens from the EASL facility and related environments were identified as to genus and species or sub-species. Of the total, 90.4-percent were gram-positive aerobic spore formers of the genus Bacillus, and 9.6-percent were gram-positive aerobic cocci, members of the genus Micrococcus and Staphylococcus, plus a Gram variable organism, Arthrobacter. The isolates were divided into seven broad groups (Groups I to VII).

A. GROUP I

Isolates in this group were identified as Bacillus megaterium. These organisms were gram-positive, short to medium rods. Colonial morphology was described as a creamy, mucoid, off-white growth. Some cultures exhibited a wrinkled appearance. Of the 21 isolates included in the group, 19 exhibited subterminal spores; the sporangia were not swollen. Two organisms were in an asporogenous state. Nineteen isolates formed a heavy rough pellicle in high salt concentrations. Isolate number E-35 showed a lighter growth in salt concentrations, whereas isolate SP-33 was negative in 10-percent NaCl broth. Growth in such high salt concentrations would indicate the inclusion of this group with the Bacillus licheniformis and Bacillus subtilis species. The biochemical reactions, however, of nitrate reduction, starch hydrolysis, and acetylmethylcarbinol productions warrant its identification as Bacillus megaterium. Nitrate reduction was uniformly negative for the group. Starch hydrolysis separated the group into subspecies A and subspecies B. Smith mentions certain strains of Bacillus cereus that were starch negative. There is a possibility this is a Bacillus cereus subgroup, although the general reactions do not indicate this fact. Growth on dextrose agar and soybean agar was similar. See Table I for the morphological and biochemical characteristics of Group I isolates.

The following key is applicable to each of Table I through VI in the following paragraphs:

pos	=	positive
neg	=	negative
pell	=	pellicle
-	=	negative test results
+	=	positive test results
mod	=	moderate growth

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP I ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	Sporulation	Spore Position	7% NaCl	10% NaCl	Nitrate	Starch	Voges Proskauer	Dextrose Agar	Soybean Agar	Tyrosine Agar	Citrate	Litmus Milk	Dextrose	Lactose	Sucrose	Genus	Species	
M-7	+	short to medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	xxx	xxx	-	xxx	sl.A	-	sl.A	Bacillus	megaterium	subsp. A
M-12	+	short to medium thick rods	mucoid	off white	pos	subterminal	pos	pos	-	+	-	mod	xxx	xxx	-	xxx	A	-	-	Bacillus	megaterium	subsp. A
M-13	+	large rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	xxx	xxx	-	xxx	A	-	sl.A	Bacillus	megaterium	subsp. A
M-14	+	short rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	xxx	xxx	-	xxx	A	-	A	Bacillus	megaterium	subsp. A
M-15	+	medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	xxx	xxx	-	xxx	A	-	A	Bacillus	megaterium	subsp. A
E-15	+	medium rods	mottled	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod to heavy	mod	-	-	pept	-	-	-	Bacillus	megaterium	subsp. A
E-23	+	large thick rods	mucoid	off white	asporogeneous state		pos pell	pos pell	-	+	-	mod	mod	-	-	A	-	-	-	Bacillus	megaterium	subsp. A
E-29	+	short to medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	mod	-	-	pept	-	-	-	Bacillus	megaterium	subsp. A
E-57B	+	short to medium rods	wrinkled	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod	mod	-	-	pept	-	-	-	Bacillus	megaterium	subsp. A
E-51	+	medium rods	wrinkled	off white	pos	subterminal	pos pell	pos pell	-	+	-	heavy	heavy	-	-	xxx	-	-	-	Bacillus	megaterium	subsp. A
SP-50	+	short thin rods	wrinkled	off white	pos	subterminal	pos pell	pos pell	-	+	-	mod to heavy	heavy	xxx	+	sl.A pept	-	-	-	Bacillus	megaterium	subsp. A
M-6	+	short to medium rods	mucoid	creamy white	pos	subterminal	pos pell	pos pell	-	-	-	scant to mod	mod to heavy	-	-	A	-	-	-	Bacillus	megaterium	subsp. B
M-8	+	short to medium rods, few pairs	mucoid	off white	pos	subterminal	pos pell	pos pell	-	-	-	scant to mod	xxx	xxx	-	xxx	-	A	-	Bacillus	megaterium	subsp. B
E-35	+	medium rods	wrinkled	off white	pos	subterminal	pos	pos	-	-	-	heavy	heavy	-	-	-	-	-	-	Bacillus	megaterium	subsp. B
E-39	+	short to medium rods	mucoid	creamy white	pos	subterminal	pos pell	pos pell	-	-	-	mod	mod	-	-	-	-	-	-	Bacillus	megaterium	subsp. B
SP-2	+	medium thin rods	mucoid	off white	asporogeneous state		pos pell	pos pell	-	-	-	heavy	heavy	-	+	sl.A	sl.A	-	-	Bacillus	megaterium	subsp. B
SP-7	+	short to medium thin rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	-	-	mod	mod to heavy	xxx	+	sl.pept	-	-	-	Bacillus	megaterium	subsp. B
SP-11	+	medium thin rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	-	-	mod	mod	xxx	-	sl.A	-	-	-	Bacillus	megaterium	subsp. B
SP-28	+	short to medium rods	wrinkled	off white	pos	paracentral	pos pell	pos pell	-	-	-	heavy	heavy	xxx	+	sl.A	-	-	-	Bacillus	megaterium	subsp. B
SP-33	+	short to medium rods	mucoid	off white	pos	paracentral	pos pell	neg	-	-	-	heavy	heavy	xxx	-	sl.A pept	-	-	-	Bacillus	megaterium	subsp. B
SP-56	+	short to medium rods	wrinkled	off white	pos	paracentral	pos pell	pos pell	-	-	-	mod to heavy	heavy	xxx	-	pept	-	-	-	Bacillus	megaterium	subsp. B

TABLE I-A

10-A

TABLE I B

-10- B

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP II ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	Sporulation	Spore Position	7 Percent NaCl	10 Percent NaCl	Nitrate	Starch	Voges Proskauer	Dextrose Agar	Soybean Agar	Citrate	Litmus Milk	Dextrose	Lactose	Sucrose	Genus	Species	
M-21	+	short to long rods	mucoid	off white	pos	paracentral	pos pell	pos pell	-	-	+	heavy	xxx	-	xxx	-	-	-	Bacillus	pumilus	subsp. A
E-1	+	short thin rods	mucoid	off white	pos	paracentral	pos pell	pos pell	-	-	+	mod to heavy	xxx	-	-	sl. A	sl. A	sl. A	Bacillus	pumilus	subsp. A
SP-13	+	short to medium	mucoid	off white	pos	paracentral	pos pell	pos pell	-	-	+	mod	mod to heavy	-	-	-	-	-	Bacillus	pumilus	subsp. A
M-1	+	medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	+	mod	xxx	-	xxx	A	A	-	Bacillus	pumilus	subsp. B
M-2	+	medium to large	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	+	heavy	xxx	-	xxx	A	A	-	Bacillus	pumilus	subsp. B
M-17	+	short to medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	+	heavy	xxx	-	xxx	-	-	-	Bacillus	pumilus	subsp. B
M-19	+	long thin single rods	mucoid	off white	pos	subterminal	pos pell	pos pell	-	+	+	heavy	mod	-	-	-	-	-	Bacillus	pumilus	subsp. B

11-A

TABLE-2-A

-11-B

TABLE - 2 - B

RE-ORDER No. 67-115

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP III ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	Sporulation	Spore Position	7% NaCl	10% NaCl	Nitrate	Starch	Voges Proskauer	Dextrose Agar	Soybean Agar	Tyrosine Agar	Citrate	Litmus Milk	Dextrose	Lactose	Sucrose	Genus	Species	
M-4	+	large, short rods in chains	mucoid	off white	pos	subterminal	pos	pos pell	+	+	-	heavy	xxx	xxx	-	xxx	-	-	A	Bacillus	firmus	subsp. A
M-5	+	medium to long rods	mucoid	off white	pos	subterminal	pos pell	pos	+	+	-	scant	xxx	xxx	-	xxx	-	-	-	Bacillus	firmus	subsp. A
M-9	+	short rods in chains	mucoid	off white	pos	subterminal	pos	pos	+	+	-	mod to heavy	xxx	xxx	-	xxx	-	-	-	Bacillus	firmus	subsp. A
M-18	+	medium thick rods	mucoid	off white	pos	subterminal	neg	neg	+	+	-	heavy	xxx	xxx	-	xxx	A	-	-	Bacillus	firmus	subsp. A
M-23	+	medium thin rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	+	-	heavy	xxx	xxx	+	xxx	-	-	-	Bacillus	firmus	subsp. A
M-24	+	medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	+	-	heavy	xxx	xxx	-	xxx	-	-	-	Bacillus	firmus	subsp. A
E-27	+	medium thin rods	mucoid	creamy off white	pos	paracentral	pos pell	pos pell	+	+	-	mod	mod to heavy	-	-	-	-	-	-	Bacillus	firmus	subsp. A
E-37	+	medium rods	mucoid	creamy off white	pos	paracentral	pos pell	pos pell	+	+	-	mod	heavy	-	-	pept	-	-	-	Bacillus	firmus	subsp. A
E-38	+	medium rods	mucoid	creamy	pos	paracentral	pos pell	pos pell	+	+	-	mod	mod	-	-	pept	-	-	-	Bacillus	firmus	subsp. A
E-47	+	short to medium rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	-	mod	mod to heavy	-	-	pept	-	-	-	Bacillus	firmus	subsp. A
E-55	+	medium to long rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	-	mod	mod to heavy	-	-	pept	-	-	-	Bacillus	firmus	subsp. A
SP-1	+	medium rods some filaments	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	-	mod	heavy	xxx	+	A pept	-	-	-	Bacillus	firmus	subsp. A
SP-2	+	short rods, few chains	mucoid	off white	asporogeneous state		pos cell	pos cell	+	+	-	mod to heavy	heavy	xxx	+	pept	-	-	-	Bacillus	firmus	subsp. A
SP-51	+	short thin rods	mucoid	off white yellowish	asporogeneous state		pos	neg	+	-	-	mod to heavy	xxx	xxx	+	-	-	-	-	Bacillus	firmus	subsp. B

12-A

TABLE-3-A

-12-B

TABLE 3-B

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MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP IV ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	Sporulation	Spore Position	7% NaCl	10% NaCl	Nitrate	Starch	Voges Proskauer	Dextrose Agar	Soybean Agar	Tyrosine Agar	Citrate	Litmus Milk	Dextrose	Lactose	Sucrose	Genus	Species
M-3	+	medium thin rods	mucoid	off white	pos	subterminal	pos	neg	+	+	+	scant	xxx	xxx	-	xxx	A	-	sl.A	B. subtilis	subsp. A
M-10	+	medium rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	+	mod	xxx	xxx	-	xxx	-	-	-	B. subtilis	subsp. A
M-11	+	short thick rods	mucoid	off white	pos	subterminal	pos	pos	+	+	+	mod	xxx	xxx	-	xxx	-	-	-		
M-16A	+	medium to long rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	+	xxx	xxx	xxx	-	xxx	-	-	-	B. subtilis	subsp. A
M-16B	+	medium to long rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	+	xxx	xxx	xxx	-	xxx	-	-	-	B. subtilis	subsp. A
M-19	+	short thin rods	mucoid	off white	pos	subterminal	neg	neg	+	+	+	xxx	xxx	xxx	-	xxx	A	-	A	B. subtilis	subsp. A
M-20	+	medium rods	mucoid	off white	pos	paracentral	neg	neg	+	+	+	xxx	xxx	xxx	-	xxx	A	-	A	B. subtilis	subsp. A
M-22	+	short thin rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	+	+	xxx	xxx	-	-	A pept	-	-	-	B. subtilis	subsp. A
E-55	+	short rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	+	mod to heavy	heavy	-	-	A pept	-	-	-	B. subtilis	subsp. A
E-28A E-28B	+	medium thin rod, some short filaments	mucoid	off white	pos	subterminal	pos pell	pos pell	+	+	+	mod	mod	-	-	casein precipitate	sl.A	-	A	B. subtilis	subsp. A
E-57A	+	short rods	mucoid	off white	pos	paracentral	pos pell	pos pell	+	+	+	mod to heavy	heavy	-	-	A pept	-	-	-	B. subtilis	subsp. A
SP-12	+	short thin rods	wrinkled	off white	pos	subterminal	pos pell	pos pell	+	+	+	heavy	heavy	xxx	-	pept	-	-	-	B. subtilis	subsp. A
SP-27	+	short thin rods	wrinkled	off white	pos	subterminal	pos pell	pos pell	+	+	+	heavy	heavy	xxx	-	sl.A pept	-	-	-	B. subtilis	subsp. A
SP-38	+	short thin rods	wrinkled	off white	asporogeneous state		pos pell	pos pell	+	+	+	scant	heavy	xxx	-	pept	-	-	-	B. subtilis	subsp. A
SP-55	+	short to medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	+	+	heavy	heavy	xxx	+	pept	-	-	-	B. subtilis	subsp. A
SP-5	+	medium rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	-	+	mod to heavy	heavy	xxx	-	A pept	-	-	-	B. subtilis	subsp. B
SP-26	+	medium rods filaments	mucoid	off white	pos	subterminal	pos pell	pos pell	+	-	+	heavy	heavy	xxx	+	A pept	-	-	-	B. subtilis	subsp. B
SP-54	+	medium rods	mucoid	off white	asporogeneous state		pos pell	pos pell	+	-	+	heavy	heavy	xxx	+	neg	-	-	-	B. subtilis	subsp. B
SP-19	+	medium rod filaments	mucoid	off white	asporogeneous state		pos pell	pos pell	+	-	+	heavy	mod	xxx	+	neg	sl.A	-	A	B. subtilis	subsp. B
SP-31	+	medium rods, some filaments	rhizoid growth	off white	pos	subterminal	pos pell	pos pell	+	-	+	heavy	heavy	xxx	+	sl.A	sl.A	-	A	B. subtilis	subsp. B
SP-32	+	short to medium thin rods	mucoid	off white	pos	subterminal	pos pell	pos pell	+	-	+	mod	heavy	xxx	+	sl.A	sl.A	-	sl.A	B. subtilis	subsp. B

13-A

TABLE 4-A

-13- B

TABLE 4-B

RE-ORDER No. 67-115

[redacted]

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP V ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	Sporulation	Spore Position	7 Percent NaCl	10 Percent NaCl	Nitrate	Starch	Voges Proskauer	Dextrose Agar	Soybean Agar	Tyrosine Agar	Citrate	Litmus Milk	Dextrose	Lactose	Sucrose	Genus Species
E-13	+	medium thin rod	mucoid	orange	pos	sub-terminal	pos pell	pos pell	-	+	+	mod	Sl. to mod	-	-	pept	sl.A	-	-	Bacillus globigii
E-14	+	medium thin rods	mucoid	orange	pos	sub-terminal	pos pell	pos pell	-	+	+	mod	mod	orange pigmentation	-	casein precipitate	-	-	-	Bacillus globigii
E-58	+	medium rod	mucoid	orange	pos	sub-terminal	pos pell	pos pell	-	+	+	mod	mod	blackening of agar	-	xxx	-	-	-	Bacillus globigii
SP-53	+	med to long rods	mucoid	sl. orange	pos	para-central	pos pell	pos pell	+	-	+	heavy	heavy	-	+	casein precipitate	-	-	-	Bacillus globigii

Note: Isolates E-13, E-14 and E-58 differ from the reactions of B. subtilis in their inability to reduce nitrate. Isolate SP-53 differs in its negative starch hydrolysis.

14-A
TABLE 5-A

-14- B
TABLE 5-B

RE-ORDER No. 67-115

[redacted]

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF GROUP VI ISOLATES

Code No.	Gram Reaction	Cellular Morphology	Colonial Morphology	Pigmentation	7 Percent NaCl	10 Percent NaCl	Nitrate	Starch	Voges Proskauer	Citrate	Litmus Milk	Catalase	Coagulase	Dextrose	Mannitol	Lactose	Sucrose	
E-21A	+	Cocci	Smooth Moist	White	pos	pos	+	-	+	+	A pept	+	+	A	sl. A	A	A	Staphylococcus aureus var. albus
E-21B	+	Cocci	Smooth Moist	White	pos	pos	+	-	+	+	A pept	+	+	A	sl. A	A	A	Staphylococcus aureus var. albus
E-2A	+	Cocci	Smooth Moist	White	pos	pos	-	-	+	-	sl. A	+	±	sl. A	-	sl. A	A	Staphylococcus epidermidis
M-25	+	Cocci	Smooth Moist	White	pos	pos	+	-	-	-	-	+	-	A	-	-	A	Staphylococcus epidermidis
E-52	+	Cocci	Smooth Moist	White	pos	pos	+	-	-	-	-	+	-	A	-	-	-	Staphylococcus epidermidis
E-6	+	Cocci	Mucoid Smooth	Yellow	pos	pos	-	-	-	-	-	+	-	sl. A	-	-	sl. A	Micrococcus luteus
E-24A	+	Cocci	Mucoid Smooth	Yellow	pos	pos	-	-	-	-	-	+	-	-	-	-	-	Micrococcus luteus
E-24B	+	Cocci	Mucoid Smooth	Yellow	pos	pos	-	-	-	-	-	+	-	-	-	-	-	Micrococcus luteus
E-9	+	Cocci	Mucoid Smooth	Red	xx	xx	-	-	-	-	sl. A	+	-	A	-	-	-	Micrococcus rhodochrous

Note: Reactions in this group are clear-cut. Isolate E-2A was the only organism which did not given an immediate positive or negative result in the biochemical tests performed.

-15- B

TABLE VI - B

15 - A

TABLE VI - A

heavy = heavy growth

xxx = no data

pept = peptonization

sl = slight

A = acid

Al = alkaline

Tyrosine agar: The (-) on Tyrosine agar indicates it did not blacken the medium; general growth was moderate.

Asporogeneous state = organism sporulated poorly at particular time slide was made. This does not imply that good sporulation is not possible.

B. GROUP II

The five isolates of this group were classified as Bacillus pumilus, subspecies A; and Bacillus pumilus, subspecies B. Gram reactions for the group were positive. Cellular morphology ranged from short to long rods. Spores were paracentral in subspecies A, and subterminal for subspecies B. The sporangia were not swollen. Nitrates were not reduced.

Subspecies A, which exhibited typical Bacillus pumilus reactions, did not hydrolyze starch, whereas subspecies B did produce starch hydrolysis. Acetylmethylcarbinol was produced by all isolates in the group. Moderate to heavy growth was observed on dextrose and soybean agar. Reactions in litmus milk and sugar fermentations were of negligible value in determining species.

See Table II for the morphological and biochemical characteristics of Group II isolates.

C. GROUP III

Isolates of this group were identified as Bacillus firmus, subspecies A and Bacillus firmus, subspecies B. Cellular morphology was varied and included short, medium and long rods with chain and filament formation. Colonial morphology exhibited an off-white mucoid growth. Spores were subterminal and paracentral. The sporgangia were not swollen. All cultures with the exception of isolate M-18 recorded positive growth in 7-percent and 10-percent NaCl broth. The ability of this organism to grow in high salt concentrations is not consistent with most reports of Bacillus firmus, but results obtained from nitrate reduction (positive), starch hydrolysis (positive), and acetylmethylcarbinol

production (negative), indicate these isolates are members of the Bacillus firmus genus and species. The moderate to heavy growth on 1-percent dextrose agar was typical of Bacillus firmus. The possible explanation for the reaction is the use of trypticase soy agar rather than nutrient agar for the base medium. Litmus milk was uniformly peptonized in the entire series.

See Table III for morphological and biochemical characteristics of the Group III isolates.

D. GROUP IV

Spores demonstrated were subterminal or paracentral. The sporangia were not swollen. Cellular morphology consisted of short to medium thin rods. Visually, the colonies appeared mucoid and off-white in color. Growth in 7 percent and 10 percent NaCl broth was excellent, with the exception of isolates M-3, M-19, and M-22. Nitrates were reduced by all isolates. Bacillus subtilis, subspecies A, hydrolyzed starch, whereas Bacillus subtilis, subspecies B, was starch negative. All isolates produced acetylmethylcarbinol. Growth on glucose and soy agar was uniformly moderate to heavy. Results observed with litmus milk were varied. The above reactions parallel those of Bacillus subtilis.

See Table IV for the morphological and biochemical characteristics of the Group IV isolates.

E. GROUP V

The four isolates of this group were characterized as Bacillus globigii and, possible, Bacillus globigii sub-species. Cellular morphology ranged from medium to thin gram-positive rods. Spores were paracentral to subterminal. The sporangia were not swollen. Orange pigmentation was produced on TSA.

Nitrate was reduced to nitrite by isolate SP-53, while the other members of the Group did not reduce nitrates. Starch was hydrolyzed by all members of the Group except SP-53. The Voges-Proskauer reaction was positive for all members of the Group.

See Table V for the morphological and biochemical characteristics of the Group V isolates.

F. GROUP VI

The members of this group were gram-positive cocci. They were placed in two genera: Staphylococcus and Micrococcus.

The Staphylococcus aureus, variety albus, was nitrate positive, starch negative, coagulase positive, grew in 10-percent NaCl, and fermented dextrose, mannitol,

lactose, and sucrose with the production of acid. It differed from the classical definition of the organism in that litmus milk was peptonized instead of coagulated.

Staphylococcus epidermidis was also identified. Of the three isolates, E2A was the closest to the classical definition of the organism, while M-25 and E-52 varied in their fermentation characteristics. The Micrococcus were M. luteus, M. rhodochrous, and another Micrococcus (E-24 A & B), whose species is questioned (might be a sub-species or variety of M. luteus. E-24A and B did not ferment dextrose, mannitol, lactose, or sucrose. See Table VI for the morphological and biochemical characteristic of Group VI isolate.

G. GROUP VII

Only one isolate was placed in this group: Arthrobacter globiformis (E-17).

Cultures of E-17 grown on TSA, 18–24 hours (young cultures), at 32°C were Gram negative; after 24 hours at 32°C, the cultures became gram positive or gram variable.

The young cultures were mainly rod-shaped cells (a few curved cells were found), while the older cultures (over 24 hours) exhibited marked pleomorphism, e.g., rods, coccoid cells, club-shaped cells, Y forms, X forms, V forms, * forms, and a number of larger coccoid cells (cystites). The cystites give rise to rod-shaped cells by germination.

Growth was poor in TS broth and TS agar at 32°C for 24 hours. Good growth was obtained when the organism was transferred to brain heart infusion agar and incubated at room temperature.

The colonial morphology was as follows: cream-colored pigmentation, opaque, smooth, shiny, soft, 1.5–2 mm in diameter.

IV. DISCUSSION

The identification of the EASL facility and related environments isolates was performed using the classical pure culture techniques. Bergey's Manual,[1] Topley and Wilson's Principles of Bacteriology and Immunology,[2] Smith's monograph on the "Aerobic Sporeforming Bacteria, "[5] Krassilnikov's Diagnostik der Bakterien and Actinomyceten,[4] and Cowan and Steel's Manual for the Identification of Medical Bacteria[2] were used to establish identity and classify the isolates.

The major problem encountered in this study was the classification of the genus Bacillus members. The genus Bacillus is noted for its unstable or variable sugar fermentation characteristics. Therefore, major emphasis was placed on growth characteristics in 7-percent and 10-percent NaCl broth, nitrate reduction, Voges-Proskauer reaction, starch hydrolysis, citrate utilization, sporangium shape (swollen versus non-swollen), spore location, and cell morphology. Secondary importance was attributed to the suger fermentations and other biochemical characteristics. Therefore, if an isolate was similar in major emphasis characteristics to a specific species, it was placed in that species. If the fermentation characteristics differed from the given species, it was considered a possible subspecies or variety. This approach is justified on the basis of the notorious variation of fermentation characteristics displayed by members of the genus Bacillus and the recommendations of Smith et al. [5]

Further study might reveal that the Bacillus subtilis subspecies or varieties, and others might be new species not previously described, or just subspecies or varieties of a given organism.

The presence of the genera Bacillus, Staphylococcus, and Micrococcus was not unexpected, since the E Series organisms were isolates from EASL, while the M Series were from modules, and the SP Series were spreaders. The sources of the organisms were from EASL itself, personnel, modules, components, tools, and instruments.

The most common aerobic mesophilic spore former in this study was the genus Bacillus, which is found in soil, air, and dust, and on most fomites. Thus, the presence of species in the genus Bacillus was not unexpected.

Staphylococcus aureus variety albus and epidermis, normal skin inhabitants, were not unexpected isolates. For even though workers in EASL were gowned (sterile hood, gloves, mask, and smock), considerable shedding of skin particles and microorganisms can take place from the uncovered face and neck areas. In addition, one cannot say that gowning was 100-percent effective in controlling skin shedding contamination.

Micrococcus luteus also was not an unusual isolate for this environment, since one of its normal habitats is dust. The other Micrococcus isolate species might be a microorganism previously not described, or a subspecies or variant of Micrococcus luteus.

The presence of Arthrobacter was not expected in the EASL environment, but should not be considered unusual, since the genus Arthrobacter is widely distributed in soil. The difficulty of identifying this genus was that it is pleomorphoric, having rod, coccoid, club-, V-, X-, and *-shaped forms, has questionable branching and is gram negative to gram variable (positive and negative), depending on age and on culture medium. As a result, many invesgators would consider the culture contaminated and discard it or try for a fresh isolation that would yield the same results.

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APPENDIX A

The following outline, defining the approach of Favero, was obtained from Dr. Martin Favero, U.S.P.H.S., Phoenix, Arizona.

I. IDENTIFICATION SCHEME FOR ENVIRONMENTAL ISOLATES

A. GRAM STAIN

Positive -- Rods

-- Cocci

Negative -- Rods

-- Cocci

B. GRAM POSITIVE RODS -- spore formers ..... Bacillus spp.

-- non-spore formers, grown on sporulation medium (AK) or (TAM) for 3–5 days, do not survive heat shock 80°C, 15 min. no spores ..... Corynebacterium spp. or Brevibacterium spp.

do survive heat shock: spores ..... Bacillus spp.

1. Corynebacterium spp.

Lactose: pos. or neg.

Glucose: pos. or neg.

Sucrose: pos. or neg.

Litmus milk: acid or alk, or coag. or reduced

Catalase: pos.

Gelatinase: pos. or neg.

Starch hydrolysis: pos. or neg.

Color: variable

Note: If culture is lactose positive, it is a Corynebacterium; if it is negative, it can be either Corynebacterium or Brevibacterium.

2. Brevibacterium spp.

Lactose: neg.

Glucose: pos. or neg.

Sucrose: pos. or neg.

Litmus milk: acid or alk. or coag. or reduced

Catalase: pos.

Gelatinase: pos. or neg.

Starch hydrolysis: pos. or neg.

Color:

3. Lactobacillus spp.

Catalase: negative

Strong acid reaction in litmus

C. GRAM POSITIVE COCCUS: COAGULASE POSITIVE

Vogel Johnson agar: acid

PRM salt agar: acid

Glucose: acid (phenol red base)

Mannitol: acid (phenol red base)

D. GRAM POSITIVE COCCUS: COAGULASE NEGATIVE

Vogel Johnson agar: alk., may be no growth or acid

PRM salt agar: alk., may be no growth or acid

Glucose: acid or alk. (P.R. base)

Mannitol: acid or alk. (P.R. base)

Glucose: acid (B. C. P.) anaerobic

Mannitol: no change (B. C. P.), may be acid only on top anaerobic

.....S. epidermidis

Vogel Johnson agar: no growth or alk.

PRM salt agar: no growth or alk.

Glucose: acid or alk. (P. R. base)

Mannitol: alk. (P. R. base)

Glucose: no change or acid only on top (B. C. P.) anaerobic

Mannitol: no change - B. C. P. anaerobic

..... Micrococcus spp.

E. GRAM POSITIVE COCCI: CHAINS (GROWN IN T. S. B.)

Vogel Johnson agar: no growth

PRM salt agar: no growth

Glucose: acid (P. R. base)

Mannitol: acid (P. R. base)

Litmus milk: acid coag.

Glucose: acid (B. C. P.) anaerobic

Mannitol: acid (B. C. P.) anaerobic

.....Streptococcus spp.

F. GRAM NEGATIVE RODS

Shewan, J. M., G. Hobbs, and W. Hodgkiss "A Determinative Scheme for the Identification of Certain Genera of Gram-Negative Bacteria, with Special Reference to the Pseudomonadaceae," J. Appl. Bact., 23, 3: 379–390, 1960.

G. GRAM-NEG COCCUS: oxidase positive ..... Neisseria spp.
negative ..... Micrococcus spp.

Glucose: acid (Bromocresol purple indicator) (See ref.
anaerobic (below
(for media

Mannitol: acid (Bromoscresol purple indicator)
anaerobic

..... S. Aureus

Subcommitte on Taxonomy of Staphylococci and Micrococci. Minutes of First Meeting. International Bulletin of Acteriological Nomenclature and Taxonomy. Vol. 15, No. 2, April 1965, pp. 107–108.